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Comparison of Four Procedures for Measuring Elastase Production by *Pseudomonas aeruginosa* Strains from Cystic Fibrosis Patients[1])

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Summary: Forty-five *Pseudomonas aeruginosa* strains were isolated from the sputa of cystic fibrosis patients. The elastase production of each strain was assayed in the culture supernatant using four different procedures, i.e. two immunological assays (RIA and ELISA), and two enzymatic assays, the latter employing either elastin or tetraalanine as substrate, with conductometric measurement of substrate hydrolysis. Elastase concentrations were determined from standard curves prepared with the same purified elastase, and expressed in mg of elastase per litre of supernatant. The resulting values were in the range reported in the literature, and differed greatly from one strain to another (0–230 mg/l). Linear relationships were found when assays were compared in pairs. Significant correlation coefficients were obtained ($r > 0.76$, $p < 0.001$) but the values were quite different for different assays. Thus, ELISA measurements were always from three to five times higher, and RIA results were from two to five times lower, than those from the other assays. Enzymatic assays with elastin gave higher values than those using tetraalanine. Most *P. aeruginosa* strains produce two other proteinases, alkaline proteinase and Las A protein. Both enzymes have limited elastolytic and peptidasic activities. The presence of alkaline proteinase does not result in falsely elevated elastase values, but an increase of elastase activity was observed when Las A was preincubated with elastin. Since this increase was not observed when tetraalanine was used as the substrate, the presence of Las A in the supernatants could explain the differences observed between the enzymatic assays. The assay with the synthetic substrate is therefore preferred.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen responsible for often fatal infections in patients with a variety of underlying diseases. Several secondary metabolites and extracellular protein toxins are related to its virulence (1). Among them, elastase, one of the extracellulary secreted proteinases plays a dominant role (2). This endopeptidase, which has been identified and characterized by *Morihara* and coworkers (3–5), has a broad substrate specificity and, in addition to elastin, cleaves various other proteins of biological importance (6–8).

Most strains of *P. aeruginosa* isolated from patients have been shown to produce elastase[2]) (10–12), although the quantity produced may vary greatly from strain to strain (9). Elastase production has been assessed mainly by enzymatic activity measurements. Assays to detect elastolytic activity include the quantification of clearance zones on elastin plates and the quantification of dye-labelled or radio-labelled elastin hydrolysis by culture supernatants of *P. aeruginosa*

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Pseudomonas aeruginosa elastase, EC 3.4.24.–

(13–15). Immunological assays have also been developed. Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) allow a direct evaluation of the amount of elastase in culture supernatants (12–13). It has been demonstrated that a good correlation exists between the different methods measuring elastase activities of *P. aeruginosa* strains (13).

In the present study, elastase production was quantified in forty-five *P. aeruginosa* strains isolated from cystic fibrosis patients. With a view to selecting the most reliable method, immunological (RIA and ELISA) procedures and enzymatic assays that monitor the hydrolysis of fibrous elastin or tetraalanine hydrolysis with the conductometric method, were compared.

Materials and Methods

P. aeruginosa strains, isolated from the sputa of cystic fibrosis patients were grown on trypticase soy broth as previously described (16).

P. aeruginosa elastase (specific activity: 74.2 mPU/mg protein) and alkaline proteinase (specific activity: 5.01 mPU/mg protein) were purchased from Nagase Co., Osaka, Japan. Their purities were checked by polyacrylamide gel electrophoresis according to *Laemmli* (17), and their concentrations were determined spectrophotometrically (5). *P. aeruginosa* Las A protein was a generous gift from Dr. *D. R. Galloway*, Department of Microbiology, The Ohio State University, Colombus, OH, USA.

Antiserum against *P. aeruginosa* elastase was produced in rabbits immunized intramusculary and subcutaneously with 75 µg of elastase in *Freund*'s complete adjuvant. The rabbits were boosted twice with the same amount of elastase in *Freund*'s incomplete adjuvant. They were bled 65 days after the first injection and the serum was decomplemented (56 °C, 30 min), filter-sterilized and stored at −20 °C. The whole serum was used without further purification for the ELISA assays, while affinity-purified IgG were prepared for the RIA assays. Both antibody solutions were checked for their antigenic specificity by Western-blotting experiments. ^{125}I radiolabelled IgG were obtained as previously described (9).

Elastin, extracted from bovine neck ligament and purified by alkaline treatment, was obtained from Elastin Products Co., Pacific, MO, USA. Tetraalanine was from Bachem, Bubendorf, Switzerland; its purity was checked by RP-HPLC. Bovine serum albumin and *o*-phenylene diamine were purchased from Sigma, Saint Louis, MO, USA. A solution was prepared containing 1 g/l *o*-phenylene diamine and 0.4 ml/l 30% H_2O_2 in 50 mmol/l citrate buffer pH 4.5.

All other products (salts and buffers) were of analytical grade.

Conductometry

The conductometric method was employed for quantifying enzymatic activities of supernatants, using either insoluble elastin or soluble peptide tetraalanine as a substrate. In a typical experiment, 4 ml of substrate (2 g/l elastin or 0.75 mmol/l tetraalanine), prepared in 5 mmol/l Tris-HCl pH 8.6, were injected in a temperature-regulated conductometric cell (type MCCD, Solea-Tacussel, Villeurbanne, France) at T = 30 °C. The temperature varied less than 0.01 °C during the experiments. The enzymatic reaction was initiated by addition of 50 µl of culture supernatant. Conductance changes were monitored over 15–20 min with a B-640 Wayne Kerr bridge. Analysis of data were performed as previously described (18). Activities, expressed in experimental units, were converted into elastase concentrations by means of standard curves (19).

Enzyme-linked immunosorbent assay

The ELISA was developed for quantification of elastase as described by *Hoffman* (20). Microtitre plates (Micro Elisa Dynatech) were coated with 100 µl of purified elastase at 0 to 40 µg/l in 0.05 mol/l carbonate buffer, pH 9.6, or various dilutions of culture supernatants in the same buffer. They were incubated for 2 hours at 37 °C then 16 h at 4 °C. The plates were washed three times with phosphate-buffered saline, pH 7.4 saturated with 10 g/l bovine serum albumin in phosphate-buffered saline, for 1 h at 37 °C, then washed again with phosphate-buffered saline supplemented with 0.5 g/l Tween 20. Sera diluted 1 : 50 in phosphate-buffered saline containing 0.5 g/l Tween 20 and 10 g/l bovine serum albumin were added to the coated wells (100 µl). After a 2 h incubation at 37 °C, the wells were washed five times with phosphate-buffered saline supplemented with 0.5 g/l Tween 20 and 100 µl of a 1 : 500 dilution of goat anti-rabbit IgG-peroxidase conjugate in phosphate-buffered saline containing 0.5 g/l Tween 20 and 10 g/l bovine serum albumin were added. The plates were incubated for 2 h at 37 °C and washed three times with phosphate-buffered saline supplemented with 0.5 g/l Tween 20. The ELISA was developed with the *o*-phenylene diamine solution (100 µl). After 10 min at 30 °C, the reaction was stopped with 50 µl of 100 g/l sodium dodecyl sulphate. The absorbance was read at 490 nm (Dynatech Minireader II, Denkendorf, Germany) and the elastase concentration was deduced from the standard curve given in figure 1.

Radioimmunoassay

The RIA was performed as described previously (15). Briefly, microtitre plates were covered with purified rabbit antielastase IgG, then incubated for 2 h at 37 °C, and overnight at 4 °C. They were washed with phosphate-buffered saline three times and saturated with 50 g/l bovine serum albumin in phosphate-

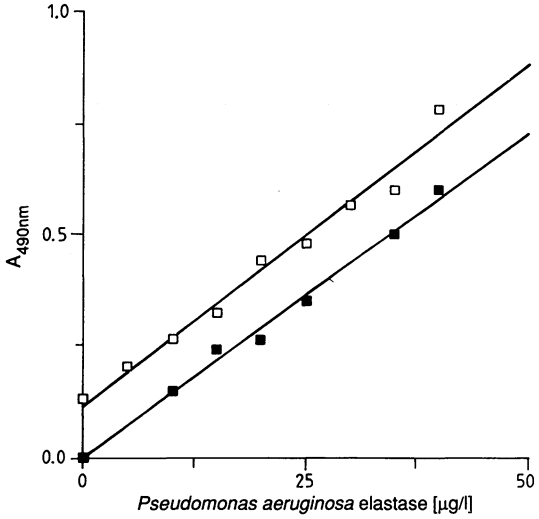

Fig. 1. ELISA standard curves.
Assays were performed as described in Material and Methods. Commercial elastase concentrations were kept between 0 and 40 µg/l. Linear relationships were obtained under the following conditions: (−■−) without supernatant, (−□−) in presence of a *P. aeruginosa* culture supernatant aliquot.

buffered saline for 4 h at 37 °C. The plates were then rinsed three times with phosphate-buffered saline, followed by the addition of 100 µl of antigen dilutions (purified elastase or culture supernatants) and incubation overnight at 4 °C. The plates were then washed three times with phosphate-buffered saline and saturated with 50 g/l bovine serum albumin. One hundred microlitres of rabbit anti-elastase [^{125}I]IgG were added, and the plates were incubated overnight at 4 °C. The wells were washed with phosphate-buffered saline and transferred to a Berthold multicrystal gamma counter (LB 2101). Elastase concentrations were calculated from a calibration curve (9).

Results and Discussion

Four assays were performed to evaluate elastase production by forty-five *P. aeruginosa* strains isolated from cystic fibrosis patients. Both immunological assays allowed a quantification of the elastase protein. Elastase concentration was also deduced from the enzymatic activities, which were determined with the aid of conductometric procedures. After each assay, the experimental data were compared with corresponding standard curves established with commercial elastase. Each value was the mean of two or three independent assays (SD was less than 10%).

The results of the assays are listed in table 1. It may be noticed that in all procedures the elastase concentrations varied considerably from one strain to another, but were in the range of the values reported in the literature.

Correlations between the different assays are shown in figure 2. A linear relationship was demonstrated in all cases, with a correlation coefficient (r) always greater than 0.76 ($p < 0.001$). The highest correlation coefficient (0.92) was obtained by comparison of the two conductometric assays. When the ELISA results were compared with those calculated from other assays, r was always higher than 0.80. On the other hand, the correlation coefficients of RIA data with conductometric data were lower ($r < 0.80$).

Correlation studies never gave a slope value of 1 which would have indicated a total agreement between the results from two different assays. In fact, big discrepancies existed between the slopes. For example, elastase concentrations from the ELISA were 3–4 times higher than concentrations deduced from enzymatic activities. In order to explain the observed differences complementary experiments were carried out: standard curves were constructed with increasing concentrations of purified elastase in the presence or absence of a defined supernatant concentration. Conductometric calibration graphs have already been published (19) whereas the ELISA calibrations are reported in figure 1. For both procedures it was shown that the slope of standard curves obtained in presence of supernatant never differed from the reference by more than 10%. These results indicate that the culture medium has no influence on the elastase assays. Another explanation may be given: the elastase concentrations were underestimated in the activity assays because deactivation and/or partial auto-digestion of the enzyme produced higher titres of antigen than of active enzyme. On the other hand, the RIA values

Tab. 1. Elastase concentration of forty-five *P. aeruginosa* strains culture supernatants isolated from cystic fibrosis patients.
Determination was performed by means of four different procedures described in Material and Methods.

Supernatant	Elastase concentration (mg/l)			
	Enzymatic assays		ELISA	RIA
	with elastin	with tetra-alanine		
1	0	0	0	0
2	50	58	208	21.5
3	0	0	5.5	0
4	32.5	35.5	132	29
5	17	25.5	57	23
6	69	74	202	16
7	45.5	44	226	29
8	33.5	42	129	21.5
9	17	14	11	13
10	50	30	183	25
11	0	2	8	2
12	55.5	44.5	215	30.5
13	18	14	85	26.5
14	28.5	17.5	30	29
15	58.5	38.5	287	34
16	16.5	8	45	6
17	54	28	176	33.5
18	40	22	–	26
19	0	0	3.5	0
20	0	0	3.5	0
21	59.5	55	123	23
22	16	0	0.5	0
23	0	0.5	7.5	2
24	22.5	14.5	51	7
25	32.5	19.5	79	5.5
26	0	0	8	2.5
27	0	0	7	1
28	13	9	54	25.5
29	0	0	1.5	1
30	52	45.5	255	–
31	0	0	8.5	0
32	42	24.5	34.5	11.5
33	0	0	17	1.5
34	0	0	3	1
35	2.5	2	14	1.5
36	12.5	1	8	2
37	0	0	1.5	0.5
38	0	0	2	0.5
39	45	43.5	42	28
40	31	33	89	25
41	28.5	39.5	71	23
42	0	0	0.5	0
43	33	40	230	37.5
44	20.5	13	–	22.5
45	0	0	0	0

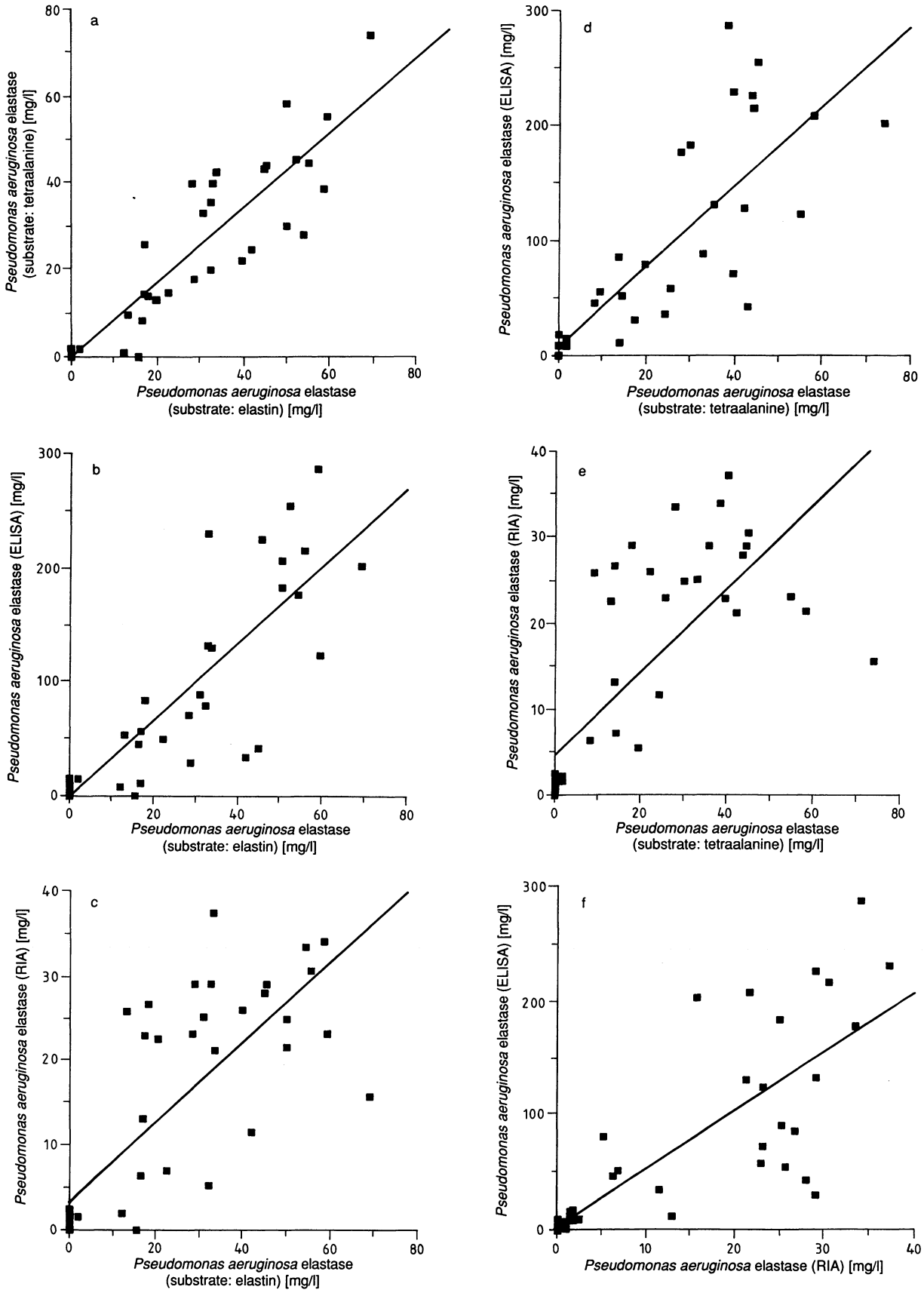

Fig. 2. Comparison of four *P. aeruginosa* elastase assays.
Linear relationships between pairs (n = 45) were demonstrated as shown by following equations:

a	y = 0.86 x − 0.47	r = 0.92	d	y = 3.49 x + 7.23	r = 0.84
b	y = 3.38 x − 1.10	r = 0.82	e	y = 0.49 x + 4.59	r = 0.76
c	y = 0.47 x + 3.21	r = 0.79	f	y = 5.12 x + 2.12	r = 0.80

were respectively two and five times lower than the conductometric and ELISA values. Differences in immunological assays may be explained by the use of different antibody preparations, i. e. whole serum for the ELISA and an IgG fraction for the RIA. The slope value obtained by comparison of the two conductometric measurements was found to be equal to 0.86, indicating a satisfactory agreement between the enzymatic assays. Nevertheless, activities monitored with elastin as the substrate were mostly higher than those measured with tetraalanine.

For a better understanding of the difference between the two conductometric results, complementary experiments were performed. Recently, *Peters & Galloway* (21) reported that another proteinase was produced by *P. aeruginosa*, the Las A protein. They demonstrated that this enzyme is able to potentiate the activity of the elastase. This assertion was supported by experiments measuring elastin-Congo red hydrolysis after a 2 h enzyme-substrate incubation. With a purified sample of Las A, we have recently performed preliminary experiments under initial-rate conditions, with either tetraalanine or insoluble elastin as the substrate. These measurements showed that the Las A protein attacks elastin very slowly compared with elastase, and it shows no activity towards tetraalanine. But when both enzymes were added simultaneously to an elastin suspension, the elastase activity was enhanced about two-fold, the elastase/Las A ratio being about 2. This phenomena was not observed when the synthetic substrate was used. If, as supposed, Las A was present in the supernatants of bacterial cultures, these observations may explain why measurements with elastin were overestimated. In addition, the capacity of *P. aeruginosa* alkaline proteinase to interfere in the conductometric assays seemed to be negligible, because 1. this enzyme cannot cleave elastin (3), and 2. its activity on tetraalanine is about 25 times lower than that of elastase. Furthermore, we demonstrated that it has no amplifying effect on the elastolytic activity of elastase under initial-rate conditions (the first thirty minutes of reaction).

Conclusion

In this study four different assays were compared to determine elastase production by *P. aeruginosa* strains isolated from cystic fibrosis patients. Immunological methods gave an estimate of the total elastase present in the supernatant, irrespective of whether the enzyme was active or inactive (deactivation, auto-hydrolysis). The conductometric procedures, however, measured only the total activity of the sample. The methodologies were therefore complementary.

The replacement of elastin by tetraalanine, an elastase-specific substrate, resulted in only minor changes in the conductometric values of activities, and both procedures correlated quite well. With this soluble synthetic substrate, the assay was easy-to-perform and reproducible values of initial velocities were obtained within thirty minutes. As previous results (15) and present ones indicate that the elastase production varies significantly among strains, this methodology may be important for a rapid quantification of elastase activity. The recent development of commercially available conductometric cells renders the method even easier. Furthermore, the current synthesis of new substrates, which are more specifically and more quickly cleaved by *P. aeruginosa* elastase, favours the proposal of the conductometric method for assaying the enzyme activity in supernatants.

This precise and reliable enzymatic assay would be highly useful if a cystic fibrosis therapeutic approach using *P. aeruginosa* elastase inhibitors were to be developed. The level of the elastase production in cultures would be an indication of the state of infection.

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